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Applicants: Harriet L. Robinson, Ellen F. Fynan, Robert G. Webster and Shan Lu

Serial No.: 08/187,879 Group Art Unit: 1804

Filed: January 27, 1994 Examiner: C. Hogue

Title: IMMUNIZATION BY INOCULATION OF DNA TRANSCRIPTION UNIT

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to Assistant Commissioner for Patents, Washington, D.C. 20231,

on 2-28-96 Elizabeth M. Mahoney
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Elizabeth M. Mahoney
Typed or printed name of person signing certificate

DECLARATION UNDER 37 C.F.R. 1.132 OF
DR. HARRIET L. ROBINSON

The Assistant Commissioner
For Patents
Washington, D.C. 20231

Sir:

Transmitted herewith is an executed Declaration Under 37 C.F.R. § 1.132 of Dr. Harriet L. Robinson and Appendix A to Declaration Under 37 C.F.R. § 1.132 of Dr. Harriet L. Robinson. On February 7, 1996, an unexecuted Declaration

and Appendix A along with a three-month Petition For Extension of Time with the appropriate fee and an Amendment with Exhibits 1-5 in response to the Office Action mailed from the Patent and Trademark Office on August 7, 1995 were filed in above-referenced application.

If the Examiner believes that a telephone conversation with Applicants' Attorney would be helpful in expediting the prosecution of this application, the Examiner is requested to call Applicants' Attorney at (617)861-6240.

Respectfully submitted,

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APPENDIX A

TO DECLARATION UNDER 37 C.F.R. 1.132

OF DR. HARRIET L. ROBINSON

#15
08/187,879

(45 pages total)

MATERIALS AND METHODS

Vaccine DNAs: The pSIV239.dpol vaccine DNA was created by cloning the SIV239.dpol insert into the pBC12/CMV expression vector. pBC12/CMV uses approximately 750 bp from the cytomegalovirus immediate early promoter to drive transcription, and sequences from the rat preproinsulin II gene to provide a 3' intron and polyadenylation signal (10). The SIV239.dpol insert (Figure 1) was generated from two separate plasmids representing the 5'(p239SpSp5') and 3'(p239SpE3') halves of a 239 provirus with a premature stop codon in *nef* (20,30). The 5' half was rendered *pol* defective by deletion of a 754 bp *Bst*EII fragment (bp 3571-4325). This deletion removed 251 codons in *pol* and introduced a stop codon at the religated *Bst*EII site. *Nar*I was used to truncate LTR sequences in the 5' proviral piece and *Stu*I to truncate LTR sequences in the 3' piece. A *Nar*I to *Sph*I fragment of the *pol*-defective 5' proviral fragment, a *Sph*I to *Stu*I fragment of the 3' half, and a blunted *Bam*HI to *Hind*III fragment of pBC12/CMV were ligated together to produce pSIV239.dpol.

The other four vaccine plasmids were constructed by cloning polymerase chain reaction (PCR)-amplified fragments of SIV *env* sequences (Figure 1) into the pJW4303 expression vector. pJW4303 is modeled on vectors described by Chapman *et al* (7). It uses approximately 1600 nt from the cytomegalovirus immediate early promoter to drive transcription (nt 458 to 2063, Genbank #M60231), and sequences from the bovine growth hormone to provide a polyadenylation signal (nt 2148 to 2325, Genbank # M57764). The vector includes a synthetic mimic of the tissue plasminogen activator leader sequence (ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCA GTCTTCGTTTCGGCTAGC) that can be placed in frame with expressed proteins using a *Nhe*I site (underlined in sequence). Templates used for PCR amplifications included p239SpE3' (SIV239), pAbT4593 (SIV251) and pL/239E/V-3 (SIV316) (48,30). The 5' PCR primer was designed to clone *env* fragments in frame with the tissue plasminogen activator leader using the *Nhe*I site. The 3' primers were designed to introduce stop codons for the production of secreted gp110 (sgp110) or gp130 (sgp130) forms of Env and to facilitate cloning into the *Bam*HI site of pJW4303. The 5' primer used for constructions was japcr19: GTCGCTCCAAGCTTGCTAGCCAATATGTCACAGTCTTTTATGG (the *Nhe*I site is underlined). The 3' primer for sgp110 was hkpcr2: CCCGGGATCctaTGCGGGCGCCAGGCCAATCGGAGTGATCTCTACTAATTT; and for sgp130, jw8: CGGGATCctaTGCGGGCGCCAGGTCAAACCAATTGCC. The *Bam*H1 site is underlined and the stop codon is in lower case in the primer sequences. Amplification was carried out in a 100 ul reaction with 2 ug of purified plasmid, 30 picomoles of each primer and 200 uM dNTPs. Five cycles of amplification were used

with 10 units of cloned *Pfu* polymerase in the buffer provided by the manufacturer (Stratagene, La Jolla CA). Each cycle consisted of 94° C for 45 sec., 56° C for 60 sec., and 72° C for 120 sec. Samples were ethanol precipitated, gel purified, digested with *Nhe*I and *Bam*HI and ligated into *Nhe*I and *Bam*HI digested pJW4303. Control plasmid DNAs consisted of pBC12/CMV and pJW4303 without inserts. Vaccine and control plasmids were grown in the HB101 strain of *E. coli* bacteria and purified twice on cesium chloride density gradients by standard protocols.

Expression of vaccine DNAs: *In vitro* expression of vaccine DNAs was tested by protein blot analyses of transiently transfected COS cells. Purified plasmid DNAs were transfected into COS cells with a Gene Pulser™ (Bio-Rad, Hercules, CA) at 960 uFD, 250 V. Two days later, cells were lysed with 10 mM Tris buffer containing 1% Triton X-100 and cell debris were removed by microcentrifugation at 12,000 rpm for 1 hr at 4°C. Aliquots of cell lysates was diluted 1:1 in 2X sample buffer (0.125 M Tris-HCl, 4.6% SDS, 10% 2-ME, 20% glycerol, pH 6.8), boiled for 2 min., cooled and loaded onto a 10% polyacrylamide gel for SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were electro-transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA). The membrane was blocked with 5% non-fat dried milk in 20 mM Tris buffer (pH 7.5) and then incubated with SIV-infected monkey sera (1:300 dilution in Tris buffer). Finally, the membrane was incubated with 5 uCi of ^{125}I-protein G in 0.5% milk/Tris buffer for 1 hr, washed with Tris buffer, air dried and subjected to autoradiography.

COS cells transfected with pSIV239.dpol or pCMV/control were examined for the production of virus-like particles by electron microscopy. At 48 hr post-transfection, cells were removed from plates with TEN buffer (40 mM Tris, 1 mM EDTA, 150 mM

NaCl, pH 7.5), pelleted in a microfuge tube and fixed in 1% glutaraldehyde. Cells were prepared for thin section electron microscopy using Osmium-Tetroxide (71).

Monkeys. Six young adult female and three young adult male Rhesus monkeys (*Macaca mulatta*), which were negative for antibodies to SIV, simian retrovirus D, Simian T-cell leukemia virus type-1, and Herpes simplex virus-1, were randomly assigned into two vaccine and one control group. Animals were individually housed and cared for at TSI Mason Inc. (Worcester, MA) according to approved Standard Operating Procedures. Animals were tranquilized for inoculations and blood collections using ketamine-HCl (10 mg/kg) as needed. Cageside observations were performed twice daily throughout the trial. Physical exams including a record of general condition, body weight, rectal body temperature and heart rate were performed at the initiation of the experiment, at weeks 11, 21 and 25 prior to virus challenge, and monthly after challenge.

Saline and gene gun inoculations of DNA: Saline injections consisted of 500 ug of each DNA dissolved in 0.5 ml saline. Intravenous inoculations were administered by mixing all of the DNAs to be delivered (0.5 ml per DNA) and performing a single injection into the brachial vein. Intramuscular injections delivered 0.25 ml of each DNA to a separate site in each quadriceps. Each gene gun inoculation consisted of 1.44 mg of 1-3 micron gold beads that had been coated with 7.2 ug of DNA by precipitation of the DNA onto beads in the presence of spermidine and Ca++ (16). Gene gun inoculations were administered to approximately 4" by 6" areas of the abdominal skin and approximately 2" by 4" areas of skin on the inner thigh. Target areas were prepared by shaving and then removing stubble with the depilatory agent, Nair (Carter Products, New York, NY). A hand-held electric discharge *Accell®* particle bombardment device (Agracetus Inc.,

Middleton, WI) was used for gene gun inoculations. Histologic analyses of tissue from target areas revealed the highest density of beads in the epidermal layer of the skin.

Virus challenge: Challenge was by intravenous inoculation into the brachial vein of 10 monkey infectious doses in one ml of tissue culture medium of uncloned SIVmac251 grown in monkey PBMC (34).

Assays for neutralizing antibody using the neutralization susceptible stock: Neutralizing antibodies to SIVmac251 and SIVsmB670 were measured in a virus-induced cell-killing assay performed in 96-well microdilution plates as described previously (45) except that CEMx174 cells were used as targets for infection. Briefly, 100 $TCID_{50}$ of virus was incubated with various dilutions of sera for 30 minutes at 37°C before the addition of cells. Neutralization was quantified by staining viable cells with neutral red. Neutral red uptake measured at A_{540} is linear from 0.25 to 1.6, corresponding from 3.1×10^4 to 2.5×10^5 viable cells per well (45). Percent protection was calculated by the difference in absorption (A_{540}) between test wells (cells + serum sample + virus) and virus control wells (cells + virus) divided by the difference in absorption between cell control wells (cells only) and virus control wells. Assays were harvested when cytopathic effect in virus control wells was greater than 70% but less than 100%. Neutralizing titers were defined as the reciprocal of the serum dilution that protected 50% of cells from virus-induced killing. $TCID_{50}$ was calculated as described previously (45).

Neutralization susceptible stock: Virus stocks for neutralization assays were harvested from cultures of acutely infected H9 cells. Stocks were clarified by low-speed centrifugation and filtration through 0.45-um pore size cellulose-acetate filters (Millipore Corp., Bedford, MA), and stored at -70°C until use. The stocks of SIVmac251 and

SIVsmB670 used to titer neutralizing antibodies are sensitive to neutralization in this assay (28,55).

Assays for neutralizing antibody for the challenge stock: Virus neutralization was measured in human PBMC and CEMx74 cells. Neutralization of SIV in human PBMC was measured essentially as described previously for HIV-1 (42). Briefly, cell-free virus (10-20 ng p27/ml) was incubated with diluted test plasmas at 37°C for 1 hr in triplicate wells of 96-well U-bottom plates before adding CEMx174 cells or PHA-stimulated PBMC (10^5 cells in 50 ul added per well). Heat inactivated plasmas were evaluated at a 1:30 dilution by incubating 50 ul of virus with 100 ul of plasma that had been diluted 1:20 in growth medium. An additional 6 wells containing no test sample (control wells) were included to determine the kinetics of virus replication in the absence of neutralizing antibodies. The plates were incubated for 3 hrs at 37°C after which time the cells were washed extensively with growth medium to remove the virus inoculum and test serum. Cell suspensions (25 ul) were collected every day beginning on day 2, mixed with 225 ul of 0.5% Triton X-100 and virus production quantified by p27 immunoassay as described by the supplier (Coulter Immunology, Hialeah, FL). Virus replication in control wells was linear from day 3 through 8, during which time p27 production increased from approximately 1 to 25 ng/ml. Virus production in test wells was measured at a time when production in control wells was in the range of 8-10 ng p27/ml. We have found that measurements of neutralization made during this stage of virus replication in control wells provides a high level of sensitivity and reproducibility to the assay. A positive score in this assay is $\geq$80% reduction of p27 CA production.

Growth of challenge stock for neutralization assays:

Uncloned SIVmac251 was obtained as a frozen vial of rhesus PBMC-grown, animal challenge stock that had been titrated in rhesus macaques (34). Virus taken from this vial was used directly to inoculate rhesus PBMC to generate an expanded single-passaged stock that was used for *in vitro* assays. Expansion was performed by incubating cells with cell-free virus for 1 day, removing the virus inoculum by a series of washes and then incubating the cells in fresh growth medium. Culture fluids were collected every two days, passed through 0.45-micron filters and stored at -70°C in 1 ml aliquots. A frozen aliquot from each time-point was thawed and SIV core antigen quantified by p27 CA immunoassay (Coulter Immunology Inc. Hialeah, FL). Culture fluids that contained the highest virus p27 concentrations were used for neutralization assays.

SIVmac251 gp110 ELISA: SIVmac251 gp110-specific IgG was measured by ELISA using alkaline-phosphatase-conjugated goat anti-monkey IgG (whole molecule, Sigma Chemical Company, St. Louis, MO) as described previously (55). SIVmac251 gp110 was a generous gift from Dr. Kashi Javaherian (Repligen Corporation, Cambridge, MA). Titers are reported as the reciprocal of the highest serum dilution giving an average absorbance at 405 nm greater than 0.1 and at least twice that of a negative control serum from a normal, healthy, SIV-negative Rhesus macaque.

Assay for anti-CD4 antibodies: To test for anti-CD4 antibodies in monkey sera, uninfected CEM cells (2 x 10^6/ml) were stained with a 1 to 10 dilution of the test serum for 30 min at room temp in 100 ul of phosphate-buffered saline containing 0.1% bovine serum albumen and 0.1% sodium azide (PBS-BSA-NaAz). Cells were washed with PBS-BSA-NaAz and stained with 10 ul of FITC-conjugated, anti-monkey IgG (whole molecule, Organon-Teknika-Cappel, Durham, NC). Cells were washed a final time and

then suspended in 2 ml of PBS-BSA-NaAz after which fluorescence was measured with a Coulter XL MCL flow cytometer. Fluorescence intensities were determined relative to cells stained with only the second antibody. Serum from a macaque immunized with human cell-grown SIV and containing high-titer anti-cell antibodies was used as a positive control.

Complement-mediated, antibody-dependent enhancement (C'-ADE) assay: C'-ADE of SIVmac251 infection was measured by p24 production in MT-2 cells as described previously (44). C'-ADE is given as the titer (last serum dilution to show enhancement), peak (serum dilution producing the greatest increase in p24 production) and power (p24 production at the peak divided by p24 production in the absence of test serum or complement). Measurements of titer and peak utilize a minimum 2-fold increase in p24 production as a cut-off value. The neutralization stock of SIVmac251 (see above) was used for C'ADE assays.

CTL assays: Effector cells for CTL assays were antigen restimulated peripheral blood lymphocytes (PBL) (66). To prepare effector cells, aliquots of $1x10^7$ PBL were placed in 12-well plates with $1x10^7$ stimulator cells (see below). This cell mix was cultured in 2 ml of RPMI-1640 medium containing 20% FCS (Flow Laboratories, McLean, VA). On day 3 of culture, two ml of medium containing rIL-2 (40 U/ml) (provided by Hoffman-La Roche, Nutley, NJ) was added to each culture. At day 6 of culture, dead cells were removed by Ficoll-Paque density gradient centrifugation and the viable cells assayed for cytotoxic function.

Stimulator cells were prepared by infecting B-lymphoblastoid cell lines (B-LCL) with recombinant-vaccinia expressing SIVmac *gag, env*, or the irrelevant equine herpesvirus type-I *gH* gene at a MOI of 10, for 12 hrs (66). Infected cultures were

washed and $1x10^7$ viable cells resuspended in 5 ml of 1.5% paraformaldehyde in PBS for 30 min. at room temperature, pelleted, resuspended in 5 ml of 0.2 M glycine-PBS for 15 min. at room temperature, and then maintained in fetal calf serum at 4°C until use in culture.

Effector cells were assayed for CTL activity on autologous B-LCL target cells (66). Target cells were prepared by incubation for 16 hrs. at 37°C in a 5% CO_2 atmosphere with both recombinant vaccinia-expressing SIVmac *gag, env*, or the irrelevant equine herpesvirus type I *gH* gene and 0.5 mCi/ml $Na_2{}^{51}CrO_4$ (ICN, Irvine, CA). Effector cells were incubated with ^{51}Cr-labeled target cells for 5 hrs with effector to target (E:T) ratios of 20:1, 10:1, 5:1 and 3:1. Specific release was calculated as [(experimental release - spontaneous release)/(100 % release -spontaneous release)] x 100. Spontaneous release varied from 10 to 20%.

Post challenge tests for infection: Post challenge levels of antigenemia were determined by analysis of plasma using the Coulter p27 CA antigen capture ELISA. Post challenge levels of infected peripheral blood mononuclear cells (PBMCs) were determined by limiting dilution co-cultivations. PBMC from 10 ml of whole blood were separated using Ficoll-Paque gradients. Twelve, serial 1:3 dilutions of PBMC, beginning with $1x10^6$ cells were co-cultured in duplicate with $1x10^5$ CEMx174 cells per well in a 24 well plate in 1 ml of RPMI 1640 supplemented with 10% fetal bovine serum. After 3 to 4 days of culture, 1 ml of medium was added to each well. The cultures were then split at 3 to 4 days intervals with an equal volume of fresh medium. Supernatants were collected at 21 days and stored at -70°C. Supernatant samples were assayed for p27 antigen using the Coulter antigen capture ELISA. Virus load was calculated as the minimum number of monkey PBMCs required to infect 50% of the co-culture wells.

Test for CD4+ cells: CD4+ cells were quantitated by fluorescence-activated cell sorting (FACS) of OKT4a (Ortho Diagnostics, Raritan, NJ)-stained PBMC. These are scored as % of the pre-challenge level. The prechallenge levels of CD4+ cells were established by averaging the results of analyses conducted on three independent harvests of cells from pre-challenge animals.

RESULTS

Vaccine DNAs and verification of expression.

To achieve a mimic of a live attenuated vaccine, a mixture of plasmid DNAs was constructed for use in vaccination (Figure 1). The first of these, pSIV239.dpol was designed to express non-infectious SIVmac239 particles. The purpose of the SIV239.dpol DNA was to present a broad spectrum of SIVmac proteins to the DNA-vaccinated host. pSIV239.dpol was constructed from the same sequences as used for the construction of the *nef*-defective SIV239 virus that had successfully protected against an uncloned SIV251 challenge (11). Sequences from both LTRs as well as sequences from an internal region of *pol* were deleted to reduce the potential for the generation of a replication competent retrovirus by pSIV239.dpol.

Four vaccine plasmids were constructed to express envelope glycoproteins of two T-cell tropic derivatives (SIVmac239 and SIVmac251), and one monocyte/macrophage tropic derivative (SIVmac316) of the uncloned SIV251 challenge (60,6). Three of these Env-expressing vaccine DNAs, designated SIV239.sgp130, SIV251.sgp130, and SIV316.sgp130, expressed the entire extracellular domain of Env (Figure 1B). The fourth, SIV239.sgp110, expressed the receptor binding subunit of Env (Figure 1B). In

these four plasmids, the secreted envelope glycoproteins were expressed as fusion proteins with a synthetic tPA leader sequence. This allows Env expression in the absence of Rev (7).

Verification of the expression of the vaccine DNAs was accomplished by analyses of transiently transfected COS cells (Figures 2 and 3). Protein blots of pSIV239.dpol-transfected cells revealed the expression of several SIV proteins (Figure 2A). Electron micrographs demonstrated the production of particles by the SIV239.dpol insert (Figure 3). Protein blots of cells transfected with the Env-expressing DNAs revealed the expected forms of Env in the culture medium as well as in the cell lysates (Figure 2A and B). Most of the glycoprotein expressed by the sgp130-expressing plasmids appeared to be present as an ~130 kD band, suggesting that proteolytic cleavage of the sgp130 form of Env at the SU/TM boundary is inefficient in COS cells. SIV-specific proteins and virus-like particles were not observed in cells transiently transfected with control DNA.

Trial Design

The trial consisted of two vaccine groups and one control group (1). The first vaccine group was vaccinated by gene gun, intravenous and intramuscular inoculations. The second was inoculated by gene gun only. The control group received control plasmid DNAs without inserts by all three routes. Gene gun inoculations were used in all of the monkeys because gene gun delivery of DNA to the epidermis has proved to require relatively low doses of DNA to raise responses (18,70,57,49). Intramuscular and intravenous saline injections of DNA were used in one vaccine group to test the ability of these routes of inoculation to augment responses raised by gun inoculations of skin.

Because of the lower efficiency of saline injections, larger amounts of DNA were used for intramuscular and intravenous inoculations than for the gun inoculations (18,70,57,49).

The DNA inoculation schedule was accomplished in three clusters of 2 inoculations (spaced at 2 weeks) with intervening 8-week rest periods (Figure 4). Clustered inoculations were used because such can enhance antibody responses to low levels of administered proteins (15). In the first cluster of inoculations, the vaccine groups received SIV239.dpol DNA, SIV239.sgp110 DNA and SIV239.sgp130 DNAs. For the 2nd and 3rd clusters of inoculations, both vaccine groups received the three 239 DNAs plus gene gun delivered SIV251.sgp130 and SIV316.sgp130 DNAs (see Table 1 for detail).

An intravenous challenge with 10 animal infectious doses of uncloned SIV251 was administered two weeks following the final DNA boost. This challenge time was chosen because optimal antibody responses are present at two weeks following immunization with protein subunits (for examples, see 3,22).

Antibody responses in DNA vaccinated macaques

Both ELISA and neutralizing antibodies were raised in all of the vaccinated monkeys (Figure 5). The first cluster of DNA inoculations raised ELISA responses in two of the 7 vaccine monkeys (one in each group). The second cluster of inoculations was followed by ELISA responses and neutralizing activity in all of the monkeys. Similar titers of neutralizing activity of from 1:216 to 1:768 were present in the gene gun and multiple route groups.

Anti-Env antibody responses were transient, with the titers of both ELISA and neutralizing activity falling between the 2nd and 3rd cluster of DNA inoculations. The third cluster of inoculations boosted the ELISA titers to levels similar to those achieved after the 2nd cluster of inoculations, but failed to boost the neutralizing antibody titers which continued to fall. To test whether the falling titers of neutralizing antibody reelected the appearance of anti-idiotypic antibody resembling the receptor binding site on Env, sera taken from weeks 14, 25 and 29 were tested for the ability to react with uninfected CD4+ cells. These tests were negative (data not shown) suggesting that the loss of neutralizing antibody was not caused by the appearance of anti-idiotypic antibody. The falling titers of neutralizing antibody also could not be attributed to the raising of enhancing antibodies (which could obscure neutralizing activity) because enhancing antibodies do not score in the neutralization assay.

The two control monkeys had low levels of ELISA activity for SIVmac251 gp110 which increased over time (Figure 5). Peak ELISA titers for one of the controls, 8R7, correlated with low levels of neutralization activity (Figure 5). This may reflect a generalized and non-specific activation of humoral responses by large amounts of plasmid DNA (approximately 18 mg per monkey in groups receiving DNA by three routes).

Susceptibility of challenge virus to raised antibody

Peak titers of neutralizing antibody were tested for their ability to neutralize the uncloned SIVmac251 challenge (Table 2). These assays were conducted with 1:30 dilutions of sera on CEM-X174 cells, a cell line that is favorable for the scoring of neutralizing activity, as well as on human PBMCs. Sera were compared to sera obtained

from macaques chronically infected with uncloned SIVmac251. None of the sera had significant neutralizing activity in the assay on PBMCs. The more sensitive assay on CEMx174 cells scored low levels of activity in most of the DNA-raised sera. One of the two samples from chronically infected macaques (a serum pool) also scored on CEMx174 cells.

CTL responses in DNA vaccinated macaques

CTL responses to Env were raised in all of the monkeys receiving vaccine DNAs (Figure 6). These were detected following antigen-restimulation of PBLs. Following the first cluster of inoculations, anti-Env CTL were present in 3 out of the 7 vaccine monkeys. One of these was in the group receiving DNA by three routes of inoculation, and two in the group receiving only gene gun inoculations. After the second cluster of inoculations, all of the vaccine monkeys exhibited anti-Env CTL activity. The levels of CTL activity were not further increased by the third cluster. In contrast to the anti-Env antibody responses that fell with time, the anti-Env CTL responses were largely persistent (see Figures 5 and 6).

Definitive CTL responses for Gag were seen in only two of the vaccine animals (Figure 6). Both of these monkeys were in the group receiving multiple routes of DNA inoculation. One monkey (L44) had high CTL responses to Gag following the first cluster of inoculations. This was the same monkey that had high antibody responses following the first cluster of inoculations (Figure 5).

The CTL activity for Env and Gag was both CD8-lymphocyte-mediated and MHC-restricted (75). Monkeys receiving control DNA did not have anti-Env or anti-Gag cytolytic activity (Figure 6).

Post challenge levels of infection.

Challenged animals were tested for protection against infection by examining antigenemia at two weeks post challenge. The vaccinated animals had 4 to 100 times lower levels of CA in their plasma than the control animals, with three of the vaccinated animals scoring below the lowest point on the standard curve (Figure 7). The detection of CA in the vaccinated animals indicated that the vaccine had not prevented infection. The low levels of CA in the vaccinated animals could have reflected early control of the infection by the vaccine and/or interference of vaccine-induced antibody with the antigen capture ELISA.

To more accurately evaluate levels of infection, PBMCs from the challenged animals were tested for the frequency of infected cells in a limiting dilution co-cultivation assay (Table 3). At two weeks post challenge, these assays revealed similar frequencies of infected PBMCs in the vaccinated and control animals. However, at 4 weeks post-challenge, all of the vaccinated animals had lower viral loads than at 2 weeks post-challenge, whereas only 1 of the 2 control animals had reduced its viral load. Again, at 6 weeks post-challenge, all of the vaccinated animals had still lower levels of infected lymphocytes, while only one of the two control animals had decreased its level of infected PBMCs. At this time, the overall difference in the levels of infected PBMCs in the vaccine and control groups was about 5-fold. Thus, the vaccination achieved some acceleration in the reduction of viral loads. The kinetics of this reduction were similar in both of the vaccinated groups.

Despite the early reductions in viral load, the vaccinated animals did not clear their infections. Furthermore, with time, the control group achieved similar reductions in viral load to those seen in the vaccinated macaques (see week 12, Table 3). Thus, the

DNA vaccination did not allow the vaccinated animals to achieve lower viral loads than those achieved by post-infection responses in the control animals.

Antibody responses at one month post challenge.

Despite the failure of the last cluster of DNA inoculations to boost neutralizing activity (Figure 5), the challenge infection raised high titers of neutralizing antibody for SIVmac251. At one month post challenge, these titers were similar or slightly higher in the vaccinated groups than in the control group (Table 4).

Interestingly, each of the vaccinated animals also had high titers of neutralizing activity for SIVsmB670 (Table 4). These titers were 3 to 27 times higher than the titers for SIVmac251. In contrast, the control animals had much lower levels of neutralizing activity for SIVsmB670. This suggests that the vaccination regimen had primed a broader neutralizing response than present in the challenged controls.

Post-challenge titers of ELISA antibody for Env were much higher (~100 times) in the vaccinated groups than in the control group (Table 4). This difference presumably reflects the vaccine having primed non-neutralizing, as well as neutralizing antibodies for Env.

Complement-dependent antibody enhancement.

Complement-dependent enhancing antibodies could be detected in the sera of the vaccinated animals pre-challenge; and, in the sera of the vaccinated and control animals post challenge (Table 5). On the day of challenge, both of the vaccinated groups had similar titers of enhancing antibody. These titers increased post challenge. At one month post challenge, the vaccinated and control animals had similar titers of complement-

dependent enhancing antibodies. This suggests that the higher ELISA response and broader neutralizing response of the DNA-vaccinated animals was not accompanied by higher titers of enhancing responses (Tables 4 and 5).

Post challenge CD4-cell levels and mortality.

Consistent with the failure to achieve long-term reductions in viral loads, all of the vaccine animals exhibited steadily declining CD4+ cells (Figure 8). One of the control animals maintained steady CD4+ levels despite an active SIV infection (Figures 7 and 8, Tables 3 and 4).

The trial was terminated at one year post-challenge. At this time, the three macaques in the gene-gun-only group, and one of the two control macaques (the one with the steady CD4+ level), had succumbed to AIDS (Figure 8). The second control monkey and the four monkeys in the multiple route group did not have clinical signs of AIDS at the time of euthanasia.

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Table 1. Summary of DNAs administered to different groups.

Group	No. of monkeys	DNA	Dose Level	Route	Dosing Frequency
1	4	239.dpol 239.sgp120 239,sgp140	250 µg each	Intramuscular injections at two sites for each plasmid	Once on weeks 1, 3, 11, 13, 21, and 23
			500 µg each	Intravenous injection	
		239.dpol[1] 239.sgp120 239.sgp140	7.2 µg each	Gene gun in two abdominal sites and one site in each thigh for each plasmid	
		251.spg140 316.sgp140	7.2 µg each	Gene gun in two abdominal sites for each plasmid	once on weeks 11, 13, 21, and 23
2	3	239.dpol[1] 239.sgp120 239.sgp140	7.2 µg each	Gene gun in two abdominal sites and one site in each thigh for each plasmid	Once on Weeks 1, 3, 11, 13, 21, and 23
		251 spg140 316.sgp140	7.2 µg each	Gene gun in two abdominal sites for each plasmid	Once on Weeks 11, 13, 21 and 23
3	2	*pBC12/CMV **pJW4303	250 µg* and 500 µg**	Intramuscular injections at two sites* or four sites**	Once on Weeks 1, 3, 11, 13, 21, and 23
			500 µg* and 1.0 mg**	Intravenous injection of indicated plasmids	
			7.2 µg each	Gene gun in two abdominal sites* and four abdominal sites**, one site* and two sites** in each thigh	
		pBC12/CMV	7.2 µg each	Gene gun in four abdominal sites	Once on Weeks 11, 13, 21 and 23

1. Gene gun administration of 239.dpol consisted of 4.8 ug of dpol DNA and 2.4 ug of pCrev DNA.

Table 2. Neutralizing activity of peak titers of DNA-raised sera for the uncloned SIVmac251 challenge stock

Group	Monkey	Highest serum dilution scoring on neutralization susceptible stock	Neutralization for challenge stock on:[2]	
			CEMx174 cells	PBMCs
Gene gun, i.m., i.v.	L44	216	no	no
	894	216	yes	no
	L37	216	yes	no
	8RK	648	yes	no
Gene gun	L116	216	yes	no
	8R5	216	no	no
	8AV	216	yes	no
Control	8BC	<24	no	no
	8R7	24	no	no
Uncloned SIVmac251-infected macaques	RL29	6144	no	no
	CMP-1	5832	yes	no

[1]Titer is the reciprocal of the highest serum dilution giving 50% neutralization.

[2]Sera are scored positive for neutralization if production of p27 CA was reduced by more than 80%. Assays were conducted at 1:30 dilutions of sera. RL29 is sera from a single chronically infected macaque. The CMP-1 sera was pooled from 12 chronically infected macaques. Assays were conducted on CEMx174 cells or on human PBMCs. See text and Materials and Methods for detail.

Table 3. Post-challenge levels of infected PBMCs[1]

Vaccine Group	Macaque	Infected PBMC at various weeks post-challenge, % of total			
		2	4	6	12
Gene gun, i.m., i.v.	L44	0.04	0.02	0.001	0.008
	894	0.1	0.04	0.0005	0.01
	L37	0.3	0.04	0.008	0.01
	8RK	0.7	0.1	0.01	0.01
	Ave.[2]	0.3	0.05	0.006	0.01
Gene gun	L116	0.1	0.01	0.004	0.008
	8R5	0.3	0.01	0.003	0.0009
	8AV	0.7	0.1	0.01	0.02
	Ave.	0.4	0.04	0.006	0.01
Control	8BC	0.1	0.1	0.02	0.02
	8R7	0.2	0.04	0.04	0.003
	Ave.	0.2	0.07	0.03	0.01

[1]Limiting dilution co-cultivations of PBMC with CEMx174 indicator cells were used to determine the % of infected PBMC at various times post-challenge. For details, see Materials and Methods.

[2]Ave., average value

Table 4. Neutralizing antibody and ELISA antibody at one month post challenge

Group	Macaque	Neutralizing antibody			ELISA antibody	
		SIVmac 251[1]	SIVsm B670[2]	B670/251[3]	251 ELISA[4]	251 neut/ELISA[5]
Gene gun	L44	1,944	5,832	3	8,100	0.2
i.m.,i.v.	894	5,832	17,496	3	8,100	0.7
	L37	5,832	17,496	3	24,300	0.2
	8RK	5,832	17,496	3	24,300	0.2
Gene gun	L116	5,832	17,496	3	24,300	0.2
	8R5	5,832	52,488	9	8,100	0.7
	8AV	648	17,496	27	8,100	0.8
Control	8BC	1,944	216	0.1	100	19
	8R7	1,944	1,944	1	100	19

[1]Titer of neutralizing antibody for the neutralization stock of SIVmac251. Numbers are the reciprocal of the highest dilution giving 50% neutralization of 100 $TCID_{50}$.

[2]Titer of neutralizing antibody for a stock of SIVsmB670.

[3]Titer of neutralizing antibody for SIVmac251 divided by the titer of neutralizing antibody for SIVsmB670.

[4]Titer of ELISA antibody for recombinant SIV251 gp110. Titers are the highest reciprocal serum dilution having an average absorbance reading greater than 0.1, and at least twice that of the negative control.

[5]Titer of neutralizing antibody for 251 divided by the titer of ELISA antibody for SIV251 gp110.

Table 5. Complement-Dependent Enhancing Antibody[1]

Group	Macaque	Bleed WK	C'-ADE		
			Titer	Peak	Power
gene gun, i.m., i.v.	L44	-1	<30	<30	<2.0
		14	30	30	4.9
		25	30	30	4.9
		29	>3840	30/60	5.4
	L37	-1	<30	<30	<2.0
		14	30	30	3.3
		25	30	30	3.5
		29	>3840	240	4.0
	8RK	-1	<30	<30	<2.0
		14	240	30	5.2
		25	960	30	6.0
		29	>3840	30	4.8
gene gun	L116	-1	<30	<30	<2.0
		14	30	30	4.3
		25	120	30	5.1
		29	>3840	30	4.5
Control	8BC	25	<30	<30	<2.0
		29	>3840	120	5.1
	8R7	25	<30	<30	<2.0
		29	>3840	30	5.2

[1]Complement-dependent enhancing activity was determined on MT-2 cells.

See Materials and Methods for experimental detail.

FIGURE LEGENDS

Figure 1. Vaccine inserts. Schematics of SIV239 proviral DNA (A) and the vaccine inserts (B). SIV239 open reading frames are indicated as open rectangles, closed or defective reading frames as dotted rectangles, and the tPA-like leader as a filled rectangle. Restriction sites used for the construction of the SIV239.dpol insert are indicated. For the constructs expressing secreted forms of Env, the amino acid sequences at the junctions of the tPA-like leader and introduced stop codons are indicated. The SIV239.dpol insert was expressed in pCMV/BC12. The Env inserts were expressed in pJW4303. See Materials and Methods for detail.

Figure 2. Expression of vaccine DNAs. Protein blot analyses of lysates (A) and culture medium (B) from transiently transfected COS cells. Approximately ~1/5th of the cell lysate and ~1/100th of the culture supernatant were loaded. Numbers at the tops of lanes indicate the SIVmac isolate from which inserts were prepared (239, SIVmac239; 251, SIVmac251; 316, SIVmac316) and the expressed insert (110, sgp100; 130, sgp130; dpol, non-infectious particles). C1 and C2 are lysates of COS cells transfected with a control vector without insert. Numbers at the side indicate the positions of SIV Env (gp140, gp130, and gp110) and CA (p27) proteins. SIV proteins were detected using serum from an SIV-infected macaque as the first antibody. For details, see Materials and Methods.

Figure 3. Electron micrograph demonstrating particle production by the SIV239.dpol vaccine DNA in transiently transfected COS cells. The bar at the bottom of the micrograph represents 100 microns.

Figure 4. Schematic of the design of the DNA vaccine trial.

Figure 5. Temporal antibody responses in vaccine and control groups. ELISA antibody for SIVmac251 gp110 and neutralizing antibody for the neutralization stock of SIVmac251 are presented in the indicated columns. The times of DNA administration are indicated by vertical arrows at the top of the schematic. Test groups are designated im, iv, and gun (the group receiving i.m., i.v. and gene gun inoculations of vaccine DNAs); gun (the group receiving only gene gun inoculations of DNA); and control, the group receiving control plasmid DNAs by i.m., i.v., and gene gun inoculations. Symbols for the individual monkeys are given at the right of the panels. Assays for ELISA antibody were run on 1 to 50 dilutions of test sera. Neutralizing titers are the reciprocal of the highest dilution of sera giving neutralization of 100 $TCID_{50}$ of the neutralizable stock of SIV251 on CEMx174 cells. For more detail see Materials and Methods.

Figure 6. Temporal CTL responses in vaccine and control groups. CTL responses are for antigen-restimulated lymphocytes from peripheral blood. Anti-Env CTL and Anti-Gag CTL are presented in the indicated columns. Arrows for the times of DNA administration, designations of test groups, and symbols for individual monkeys are as in

Figure 5. Specific lysis is given for an effector to target cell ratio of 20 to 1. Levels of specific lysis below 5% were not considered significant. For more detail, see Materials and Methods.

Figure 7. Antigenemia at 2 weeks post-challenge. Symbols for monkeys are the same as in Figures 5 and 6. Values preceded by < were between the lowest point of the standard curve and background.

Figure 8. Temporal levels of CD4+ cells in challenged monkeys. The groups and symbols for monkeys are the same as in Figures 5-7. The Christian crosses followed by numbers in parentheses designate the week of euthanasia for macaques that developed AIDS. For more detail see Materials and Methods.

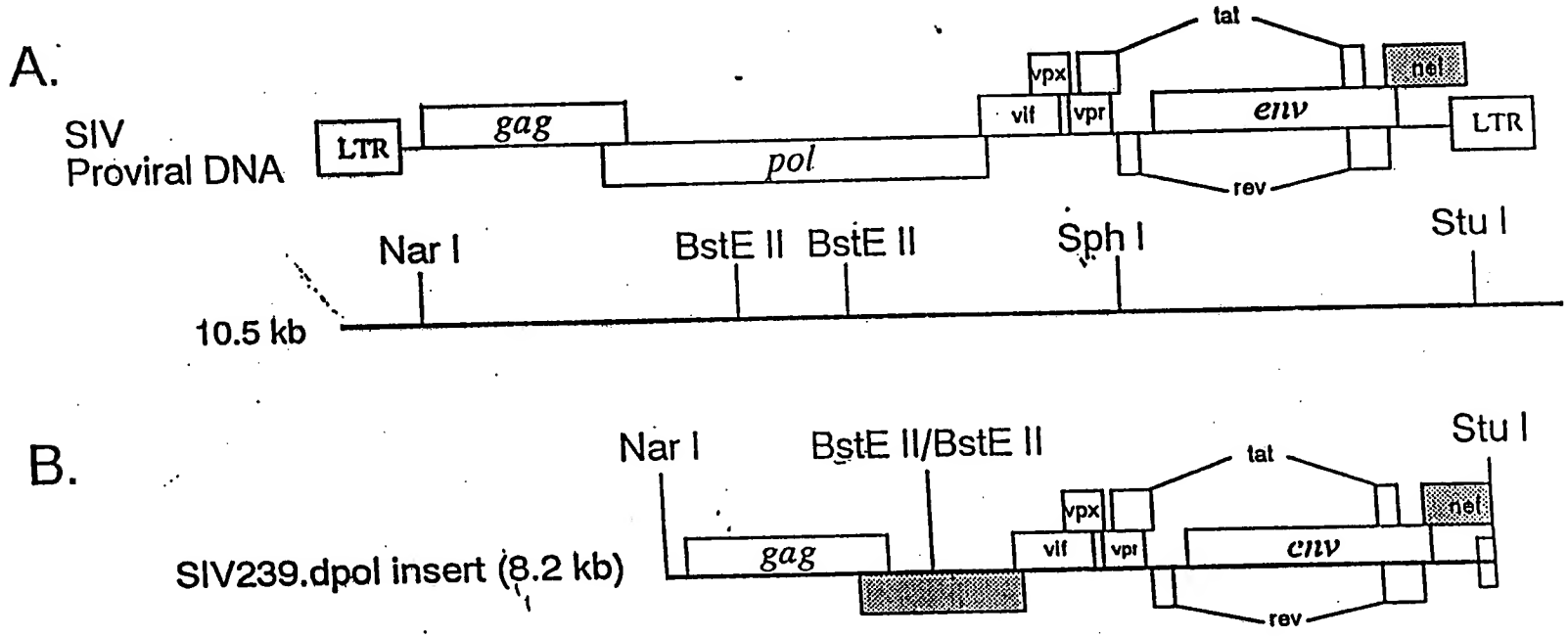
A.
SIV
Proviral DNA
LTR
gag
pol
vif
vpx
vpr
tat
rev
nef
env
LTR
Nar I
BstE II
BstE II
Sph I
Stu I
10.5 kb
B.
Nar I
BstE II/BstE II
Stu I
SIV239.dpol insert (8.2 kb)
gag
vif
vpx
vpr
tat
rev
nef
env
tPA leader-LYVT
GLAPA-stop
SIV239.sgp110 insert (1.5 kb)
NYFDL-stop
SIV239.sgp130 insert (2.1 kb)
SIV251.sgp130 insert (2.1 kb)
SIV316.sgp130 insert (2.1 kb)

Fig 1

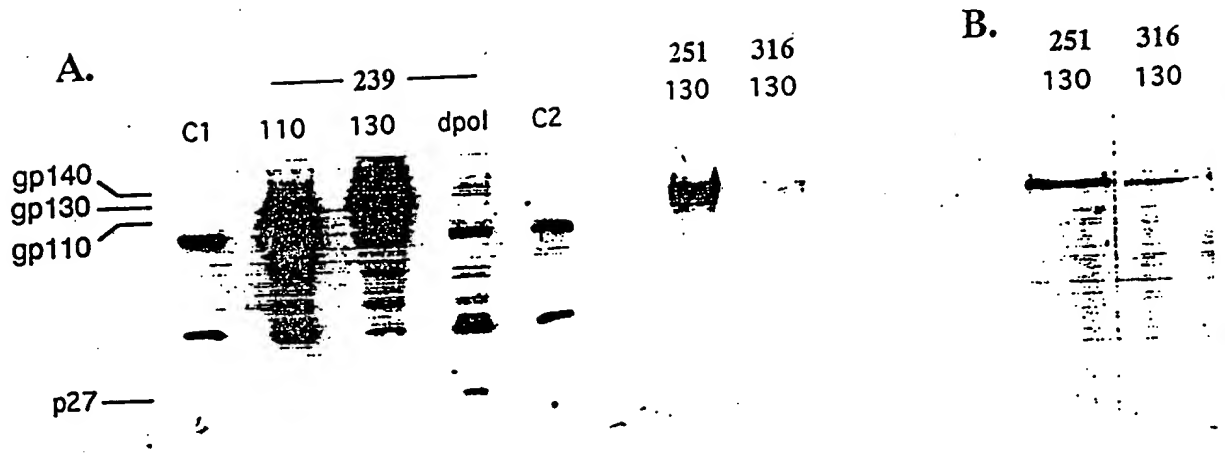

Fig 2

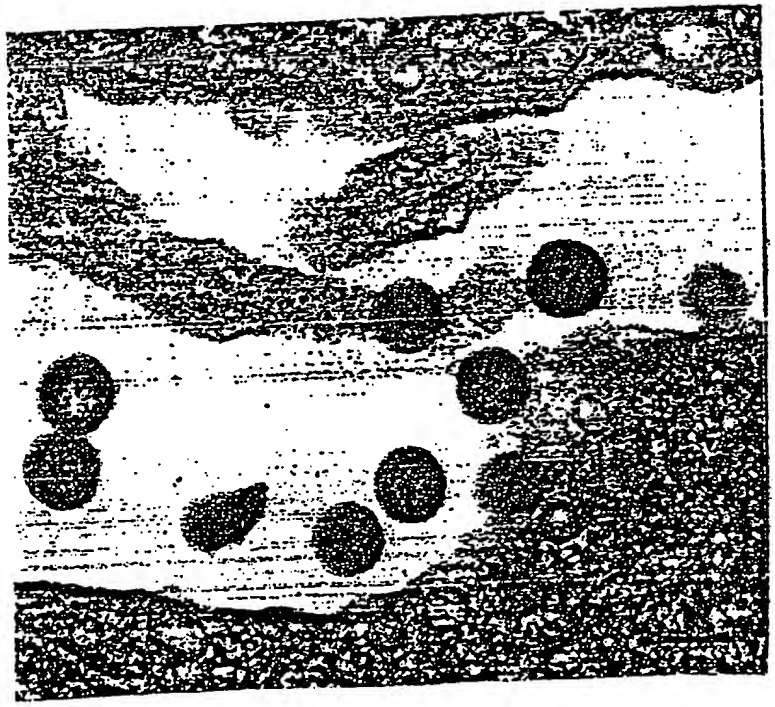

Fig 3 useful

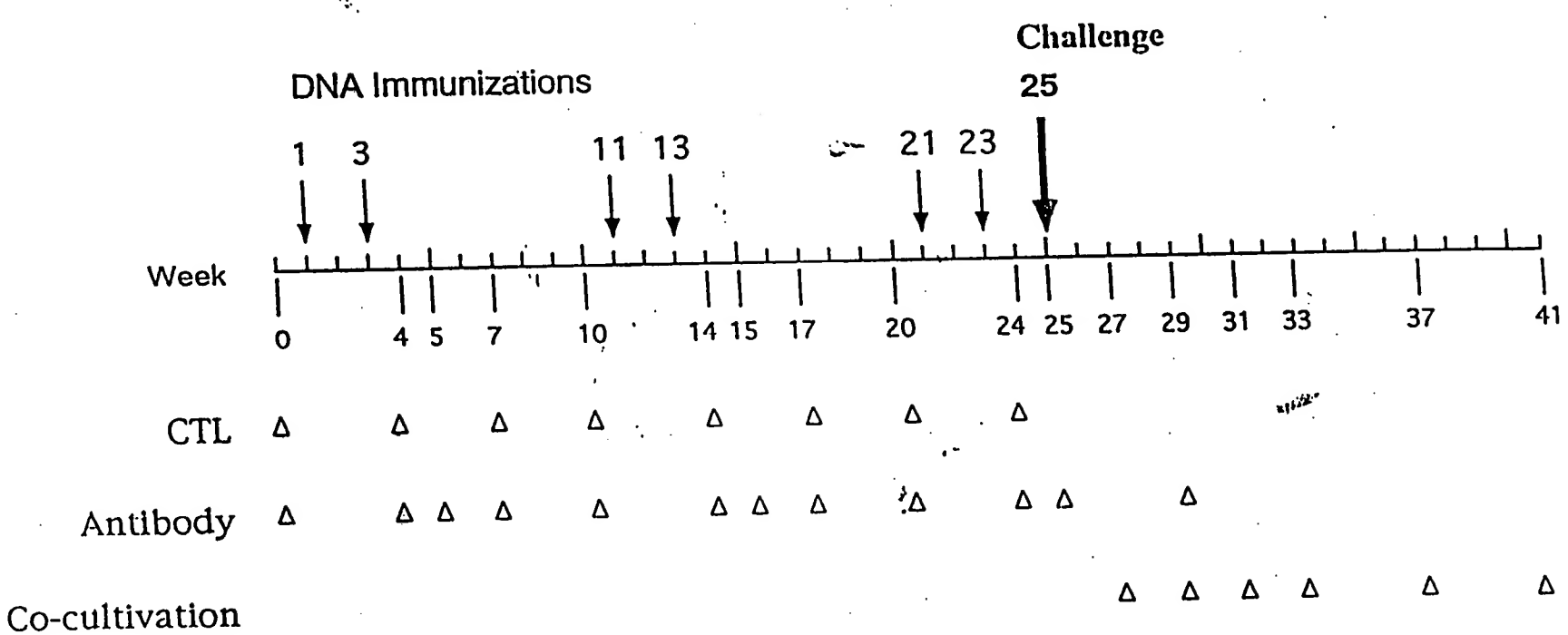
Challenge
25
DNA Immunizations
1 3 11 13 21 23
Week
0 4 5 7 10 14 15 17 20 24 25 27 29 31 33 37 41
CTL
Antibody
Co-cultivation

Fig 4

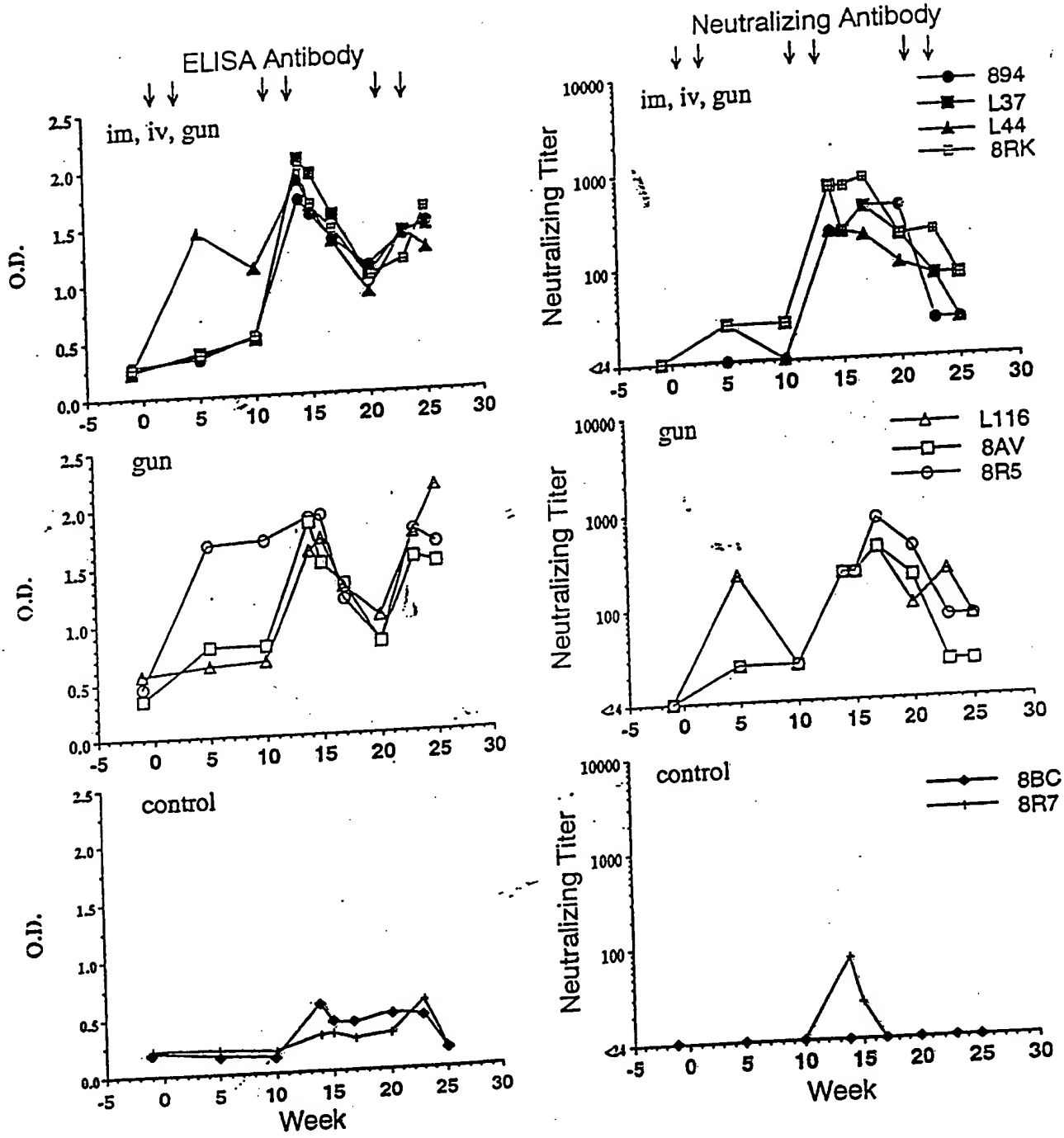
ELISA Antibody
Neutralizing Antibody
im, iv, gun
gun
control
O.D.
Neutralizing Titer
Week
894
L37
L44
8RK
L116
8AV
8R5
8BC
8R7

Fig 5

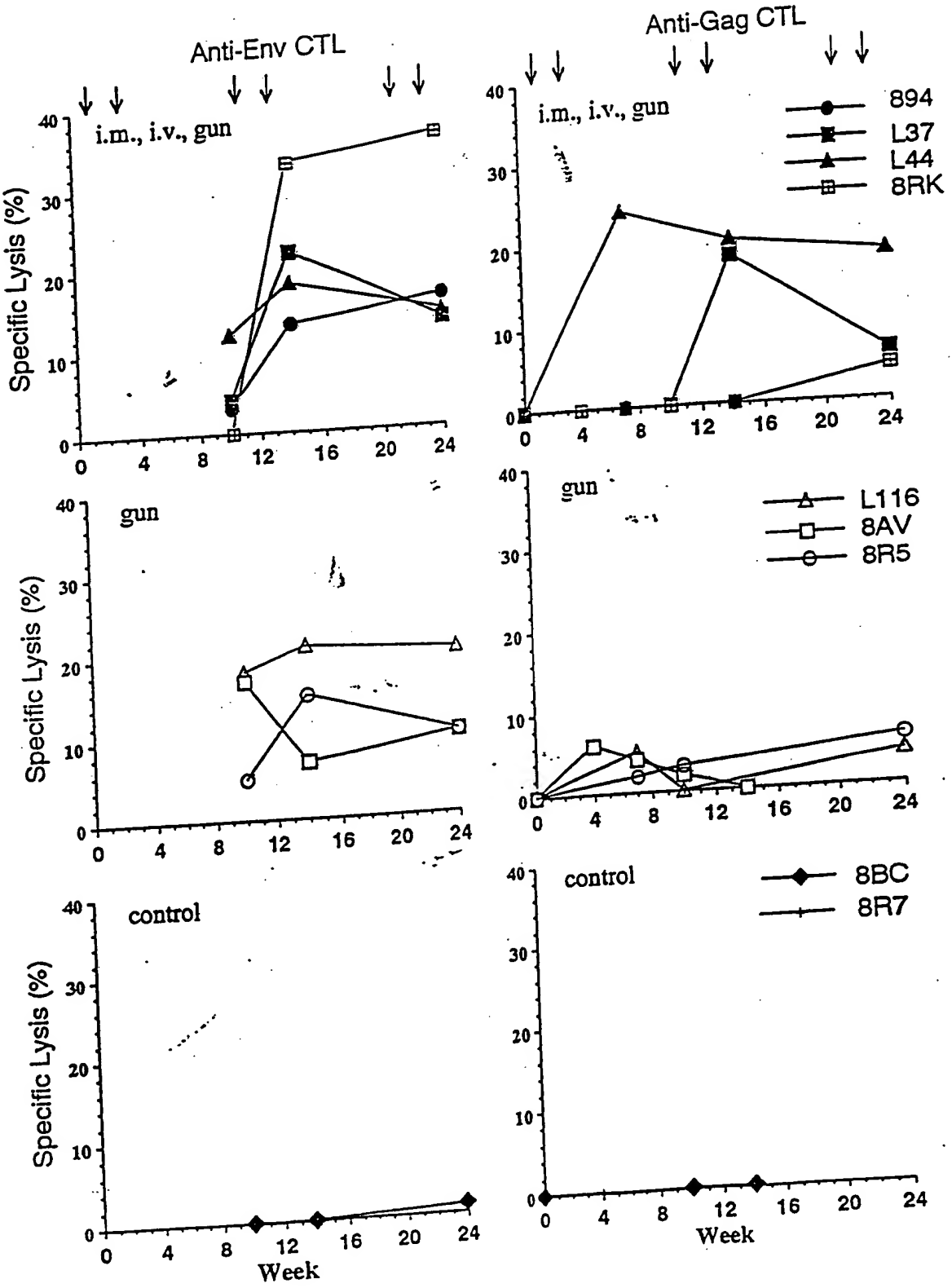
Anti-Env CTL
Anti-Gag CTL
i.m., i.v., gun
i.m., i.v., gun
894
L37
L44
8RK
Specific Lysis (%)
gun
gun
L116
8AV
8R5
control
control
8BC
8R7
Week
Week

Fig 6

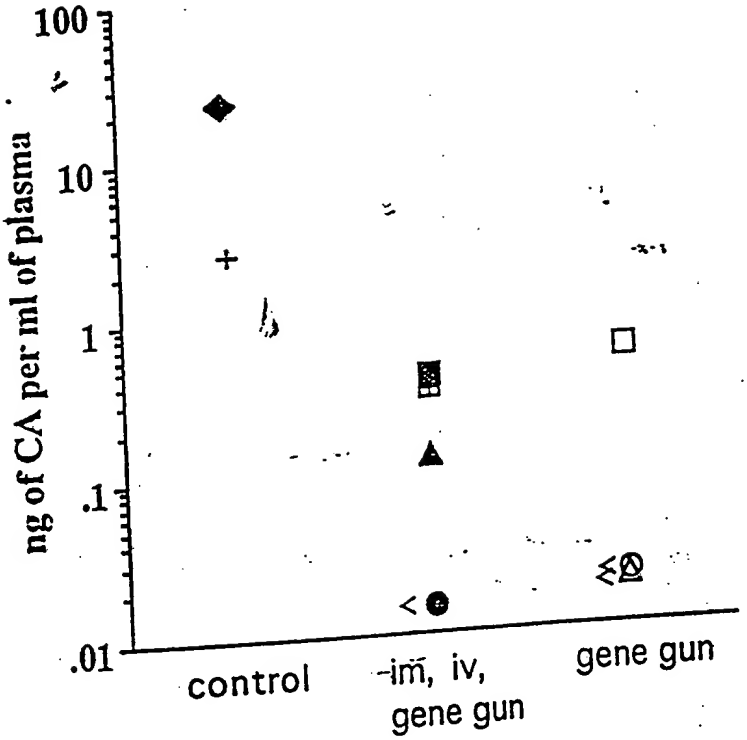
100
10
1
.1
.01
ng of CA per ml of plasma
control
im, iv,
gene gun
gene gun

Fig 1

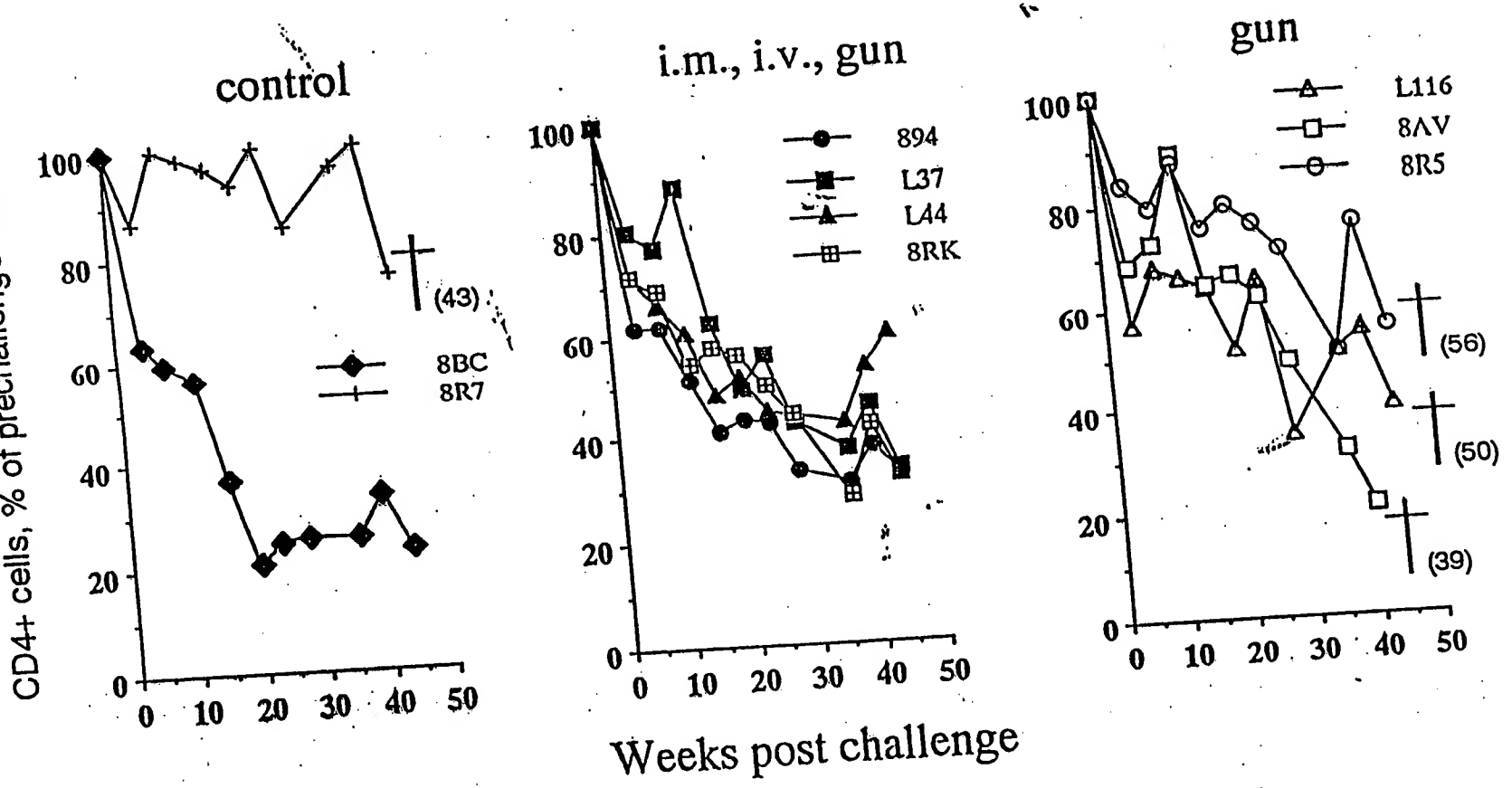
control
i.m., i.v., gun
gun
CD4+ cells, % of prechallenge level
Weeks post challenge
100
80
60
40
20
0
0 10 20 30 40 50
8BC
8R7
(43)
894
L37
L44
8RK
L116
8ΛV
8R5
(56)
(50)
(39)

Fig 8